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Supplementation of *Perkinsus marinus* Cultures with Host Plasma or Tissue Homogenate Enhances Their Infectivity

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The protozoan oyster parasite *Perkinsus marinus* can be cultured in vitro in a variety of media; however, this has been associated with a rapid attenuation of infectivity. Supplementation of defined media with products of *P. marinus*-susceptible (*Crassostrea virginica*) and -tolerant (*Crassostrea gigas*, *Crassostrea ariakensis*) oysters alters proliferation and protease expression profiles and induces differentiation into morphological forms typically seen in vivo. It was not known if attenuation could be reversed by host extract supplementation. To investigate correlations among these changes as well as their association with infectivity, the effects of medium supplementation with tissue homogenates from both susceptible and tolerant oyster species were examined. The supplements markedly altered both cell size and proliferation, regardless of species; however, upregulation of low-molecular-weight protease expression was most prominent with susceptible oysters extracts. Increased infectivity occurred with the use of oyster product-supplemented media, but it was not consistently associated with changes in cell size, cell morphology, or protease secretion and was not related to the susceptibility of the oyster species used as the supplement source.

Perkinsus marinus is a protozoan parasite of the eastern oyster *Crassostrea virginica*. Mortalities caused by this parasite typically occur in the second summer of infection and have been responsible for much of the recent decline in the oyster fishery along the eastern seaboard of the United States (3, 15). The molecular mechanisms of parasite infectivity, virulence, and interaction with the host defense system are largely unknown. The development of media formulations allowing axenic culture of *P. marinus* has provided new opportunities to assess the effects of host components on parasite growth, physiology, and infectivity. There are several media formulations for in vitro *P. marinus* culture that employ commercial base formulations (e.g., Dulbecco modified Eagle's medium with Ham's F-12 nutrient mixture) supplemented with such constituents as cod liver oil, bovine serum albumin, yeastolate, or fetal bovine serum (FBS) or its α -fetoprotein constituent, fetuin (11, 18, 19, 21, 23, 26). There is also a chemically defined, protein-free medium (ODRP-3 [25]) which has proven to be of particular value for the production of antibodies against *P. marinus* extracellular products (12).

The in vivo *P. marinus* life cycle begins with a small, immature trophozoite that enlarges over time into a "signet ring" form, so named for its large vacuole and offset nucleus. This mature trophozoite may then undergo palintomic fission, in which 4 to 64 or more immature trophozoites are formed within, then exit from, the parental cell, or tomtom, wall (35). *P. marinus* can also form motile zoospores, again by palintomic fission, with exit of the zoospores through a discharge tube and pore structure formed on the wall of the enlarged parental trophozoite, the zoosporangium (35). During in vitro culture in

ODRP-3 medium, cellular proliferation is apparently solely by binary fission, and no zoosporulation is seen. In an effort to more closely simulate, in vitro, the milieu to which *P. marinus* is exposed in vivo, oyster tissue homogenate and plasma from *P. marinus*-susceptible and -tolerant oyster species have been employed as culture supplements (20, 21, 29). Compared with growth in unsupplemented ODRP-3, *P. marinus* cells grown in the presence of plasma supplements from *Crassostrea gigas*, *Crassostrea ariakensis*, or infected *C. virginica* oysters show reduced in vitro proliferation (20). Uninfected *C. virginica* plasma supplementation, however, results in only minimal inhibition of proliferation (20, 29). Oyster tissue homogenate-supplemented medium produces marked changes in cell proliferation, morphology, and differentiation, including enlargement of trophozoites and induction of tomtom stages, which are rarely seen in unsupplemented ODRP-3 medium but are commonly observed during infection (29).

Several studies have implicated proteases as contributing factors in *P. marinus* virulence (17, 28, 32, 33). The extracellular products of *P. marinus* cells grown in ODRP-3 contain only high-molecular-mass (>50-kDa) proteases (29). However, when *C. virginica* plasma or homogenate is used as a media supplement, there is significant alteration in *P. marinus* protease expression patterns, including a simultaneous downregulation of high-molecular-mass proteases and upregulation of low-molecular-mass (<50-kDa) proteases. These changes are not seen when cells are exposed to homogenates from *C. gigas* and *C. ariakensis* oysters (29), both of which have been reported to be more tolerant of *P. marinus* infection than is *C. virginica* (2, 7, 8, 30).

It is not clear if there is an association between the observed changes in cellular morphology and the shifts in the secreted protease profiles in supplemented cultures, or whether either is associated with parasite infectivity. In order to better under-

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stand these relationships, protease production, in vitro cell size and morphology, and infectivity were assessed under various conditions. Observations were made of variations in these factors among clonal *P. marinus* isolates, and then one isolate was selected for investigation of alterations in the presence of various host-derived media supplements. A detailed observation of the effects of tissue-based supplements derived from susceptible and tolerant oysters was then performed, and a selected dosage level was used to investigate infectivity using a variety of oyster populations and species.

MATERIALS AND METHODS

Animals. Experimental oysters were maintained in 1- μ m-filtered York River (Va.) water and fed commercially produced algae (Reed Mariculture, San Jose, Calif.). Water changes were performed twice weekly, and all effluent water was chlorinated prior to release. *P. marinus*-free *C. virginica* oysters from Maine (*C. virginica* ME; Pemaquid Oyster Company, Waldoboro, Maine) were initially employed, but the occurrence of *P. marinus* infections in those oysters over the course of this study necessitated the importation of disease-free *C. virginica* oysters from Washington state (*C. virginica* WA; Taylor Shellfish Farms, Shelton, Wash.). *C. gigas* oysters were purchased from Taylor Shellfish Farms. *C. virginica* oysters originally from populations in Louisiana (*C. virginica* LA), Tangier Sound (*C. virginica* TG) in the Chesapeake Bay (Va.), and the CROSBreed program (*C. virginica* XB; selectively bred for *P. marinus* resistance) were all gathered from a single deployment site in the Yeocomico River, a tributary of the Potomac River (Va.) near its entrance to the Chesapeake Bay. *C. ariakensis* oysters were provided by the Virginia Institute of Marine Science oyster hatchery. A subsample of both the *C. virginica* ME and *C. virginica* WA oysters used in infection trials was confirmed to be *P. marinus*-free by body burden analysis (see below).

Infection trials and analysis of *P. marinus* body burden. Oysters were infected by injection of parasites into the mantle cavity through a small hole in the shell made with a lapidary saw. Each oyster was injected with 10^6 parasite cells suspended in 100 μ l of artificial seawater (20 ppt; Forty Fathoms Marine Mix; Marine Enterprises International, Baltimore, Md.) on days 1, 3, and 5, and a sham group was injected with artificial seawater. The oysters were maintained at a density of 10 to 15 per 35-liter aquarium and sacrificed after 4 weeks for analysis of *P. marinus* infection level.

In order to most accurately and sensitively quantify the infection level following experimental infection, *P. marinus* cells were enumerated using the whole body burden technique (10, 14). Briefly, the oyster soft tissues were finely minced and incubated at room temperature for 7 days in 20 ml of fluid thioglycolate medium (2.9% FTM; T-9032; Sigma-Aldrich, Inc., St. Louis, Mo.; 2% NaCl) supplemented with penicillin-streptomycin solution (5% [vol/vol]; P-0781; Sigma-Aldrich, Inc.). Following incubation, the tissue was pelleted by centrifugation, resuspended in 20 ml of 2 M NaOH, and held at 60°C for 3 to 4 h to dissolve the oyster tissue. The enlarged *P. marinus* prezoosporangia (hypnospores) were then pelleted, washed three times in distilled water, stained with a 1:5 dilution of Lugol's iodine, immobilized on a 0.45- μ m filter by vacuum, and enumerated by light microscopy. When necessary, dilutions of the hypnospores were made in distilled water and counted in triplicate, and the mean count was extrapolated to the volume of the sample. The resultant counts were log_e transformed for analysis of variance and for Tukey's multiple comparisons test, using a 5% error rate.

Analysis of proteolytic enzyme expression. Supernatants from *P. marinus* cultures and their respective media controls were analyzed for protease production by zymography. Twenty microliters of media or culture supernatant was electrophoresed under nonreducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (24) using an 8% polyacrylamide separating gel copolymerized with 0.1% porcine gelatin (G-8150; Sigma-Aldrich, Inc.) and a 4% stacking gel. Following electrophoresis, the gels were washed three times for 10 min in 2.5% Triton X-100 (BP151; Fisher Scientific, Fairlawn, N.J.) to renature the proteins and then once in 0.1 M Tris HCl (pH 8.0; BP152; Fisher Scientific). The gels were then incubated at 37°C in a fresh change of Tris HCl (pH 8.0) buffer. While use of buffers from pH 4.0 to 9.0 have previously been shown not to alter the *P. marinus* zymographic profile, pH 8.0 is optimum for detection of *P. marinus* proteolytic activities by zymogram (27) and has been specifically used for the study of the modulation of protease expression under various media supplementation schemes (29). Following incubation, the gels were stained overnight in Coomassie brilliant blue G-250 (0.1%; 161-0406; Bio-Rad, Hercules, Calif.) in 40% methanol-10% acetic acid. The gels were

destained in the same solution, without the stain. The gels were then assessed for the presence of cleared bands corresponding to the location of gelatinolytic proteases. Since the gels were, by necessity, run under nonreducing conditions, no attempt was made to assign absolute molecular masses to them. The proteases are, for convenience, referred to as high- (>50-kDa) and low- (< 50-kDa) molecular mass species.

Assessment of differences in infectivity among several isolates of *P. marinus*. *P. marinus* clonal isolates LA10-1 (ATCC 50906; genotype 4), MA2-11 (ATCC 50896; genotype 1), SC3-2 (genotype 3) (6), and P-1 (genotype 9) (13), and the isolate HVA-18 (ATCC 50764; genotype 8) (6) were cultured for use in an infection trial and were selected based on previous genotype identification at eight polymorphic loci (38). The HVA-18 isolate was not initially cloned; however, genetic analysis of this isolate at three loci showed little genetic variability, supporting the possibility that it has become clonal by serial passage (unpublished data). All cultures were seeded at 10^6 cells per ml in 75-cm² flasks containing 50 ml of a supplemented Dulbecco modified Eagle's medium-Ham's medium with 5% FBS, commonly known as Dungan's medium (11, 16). Cultures were maintained in a humidified incubator at 27°C under 5% CO₂ for 3 weeks and harvested for oyster challenge, and the culture supernatants were assayed by zymography. Prior to challenge, cells were enumerated by hemacytometer and their mean size was estimated using an ocular micrometer. Twenty *C. virginica* ME oysters per group were subjected to experimental infection.

Assessment of the effects of oyster plasma and tissue homogenate supplementation on *P. marinus* infectivity. The P-1 isolate of *P. marinus* was chosen for further study based on its demonstrated potential to alter its secretion profile of high- and low-molecular-mass proteases following media supplementation. Both plasma and tissue homogenate-supplemented media induced similar changes in P-1 protease expression, but only homogenate altered cell size and morphology, allowing the opportunity to distinguish correlations between these alterations and parasite infectivity. Hemolymph was withdrawn from the adductor muscle through a shell notch in three *C. virginica* ME oysters, using a syringe fitted with a 25-gauge needle. The hemocytes were removed by centrifugation (500 \times g, 5 min), and the plasma was pooled and filtered through a 0.22- μ m filter (μ Star LB; Costar, Corning, Inc., Acton, Mass.). The oyster tissues were pooled and processed for homogenate basically in the manner described in MacIntyre et al. (29). Briefly, the oyster soft tissue was finely minced, suspended in 10 ml of cold artificial seawater, and homogenized using a glass Tenbroek homogenizer. This homogenate was centrifuged for 30 min at 2,500 \times g at 4°C, and the supernatant was withdrawn and centrifuged at 12,000 \times g for an additional 30 min at 4°C. This partially clarified homogenate was then filtered to 0.22 μ m by syringe filters, and the protein content was assayed by the bicinchoninic acid technique (Pierce, Rockford, Ill.).

Cultures of the P-1 isolate of *P. marinus* were seeded at 10^6 cells per ml in 75-cm² flasks containing 50 ml of ODRP-3 medium without supplementation or supplemented with 0.3 mg of plasma/ml or 0.3 mg of tissue homogenate/ml. The *P. marinus* cells used in this experiment had been cultured on a long-term basis in unsupplemented ODRP-3 prior to exposure to the media supplements. Cultures were maintained in a humidified incubator at 27°C under 5% CO₂ for 4 weeks. Cells were then harvested for oyster challenge, and the culture supernatants were assayed by zymography. Prior to challenge, cells were enumerated by hemacytometer and their mean size was estimated using an ocular micrometer. Thirty *C. virginica* ME oysters per group were subjected to experimental infection.

Assessment of the effects of homogenate supplementation on *P. marinus* cell size, morphology, and life stages. In view of the restitution of infectivity and superior induction of proteases by oyster homogenate (see Results), it was elected to investigate in greater detail the effects of homogenate supplementation on the *P. marinus* P-1 isolate. Cells were cultured in ODRP-3 medium supplemented with homogenates of tissues from five individual *C. virginica* WA, *C. gigas*, and *C. ariakensis* oysters. FBS and fetuin were used as control supplements. ODRP-3 medium was supplemented at 1.0, 0.33, 0.11, 0.037, 0.012, and 0.004 mg/ml on a protein basis or was left unsupplemented. Each well was seeded with 10^6 *P. marinus* cells in 1 ml of medium in duplicated 48-well tissue culture plates. An additional set of unseeded plates were maintained as media controls. All plates were held in a humidified incubator at 27°C under 5% CO₂ for 6 weeks.

Following incubation, the cells were resuspended (30 100- μ l passages through a 200- μ l pipette tip) prior to hemacytometer counts of single and clustered trophozoites. The exact enumeration of the cells within clusters was not possible, as they were extremely adherent, and thus clusters were ranked as groups of 2 to 3, 4, 5 to 16, and greater than 16 cells. Additionally, measurements of cell diameters of 10 trophozoites were completed for each culture well using an ocular micrometer, at a magnification of $\times 400$. In order to simultaneously

compare changes in both cell volume and count, a pellet volume was calculated as the average cell volume $[(4/3)\pi r^3]$ multiplied by the estimated total cell number, with cell numbers in clusters estimated by multiplying by 2.5, 4, 10.5, and 32 times the number of clusters of 2 to 3, 4, 5 to 16, and >16 cells, respectively. The volume of the ODRP-3 controls was then subtracted from all groups to assess deviation from the control culture. Culture supernatants from all treatments were analyzed for protease activity by zymography.

Assessment of the effects of homogenate supplementation from different *Crassostrea* species and *C. virginica* populations on *P. marinus* infectivity. Oyster tissue homogenates were produced from pooled tissues of three oysters from each of the test groups, *C. virginica* LA, *C. virginica* TG, *C. virginica* XB, *C. virginica* WA, *C. gigas*, and *C. ariakensis*. Cultures of the P-1 isolate of *P. marinus* were seeded at 10^6 cells per ml in 75-cm² flasks containing 50 ml of ODRP-3 medium without supplementation or supplemented at 0.25 mg/ml with homogenates from each of the oyster groups. This supplementation level was chosen based on prior observations of alterations in *P. marinus* culture cell count, morphological characteristics, and protease production. Cultures were maintained in a humidified incubator at 27°C under 5% CO₂ for 4 weeks. Cells were then harvested for oyster challenge, and the culture supernatants were retained for zymography. Prior to challenge, cells were enumerated by hemacytometer and their mean size was estimated by measurement of 10 cells per treatment using an ocular micrometer. Thirty *C. virginica* WA oysters per group were subjected to experimental infection.

RESULTS

***P. marinus* clonal isolates display a range of infectivity and protease expression.** *P. marinus* clonal isolate cell diameters were consistent within each group, ranging from 2 μ m (SC3-2) to 4 μ m (LA10-1, MA2-11, and P-1). The cell size of the HVA-18 isolate was more variable, ranging from 2 to 6 μ m. All cells in the cultures were single trophozoites, with little to no clustering of cells. There were significant differences in the oyster *P. marinus* body burdens 4 weeks after experimental infection (Fig. 1A), with body burdens in all groups significantly greater than that in SC3-2. The LA10-1, P-1, and HVA-18 isolates were not significantly different. The MA2-11 isolate was significantly more infective than both the P-1 and the HVA-18 isolates. There were no mortalities, and all sham-injected oysters were negative for *P. marinus* infection.

Low-molecular-weight proteases were most strongly expressed in the culture supernatants of HVA-18 and MA2-11 (Fig. 1B). The LA10-1 and P-1 isolates also showed some weak low-molecular-weight protease activity. High-molecular-weight proteases were most prominent in the HVA-18 and P-1 culture supernatants. The SC3-2 culture supernatant did not have any apparent protease activity. The FBS proteins, which can be visualized as dark bands on the zymogram, were degraded to some extent in the MA2-11 and P-1 cultures and were completely degraded in the HVA-18 culture.

Infectivity of *P. marinus* isolate P-1 is increased by medium supplementation with oyster plasma or tissue homogenate. Unsupplemented cells and cells supplemented with plasma were similarly sized, with most cells being 5 to 6 μ m. The homogenate-supplemented cells were larger, at \sim 10 μ m, and had frequent multicellular tomont structures, as well as numerous clusters of adherent cells. These were not seen in the plasma-supplemented or the unsupplemented cultures. Gelatin zymograms demonstrated production of low-molecular-weight proteases in supplemented cultures, with greater activity induced by homogenate supplementation (Fig. 2B). The media controls did not demonstrate any proteolytic activity.

Infection levels at 4 weeks were significantly higher in oysters challenged with *P. marinus* from supplemented cultures

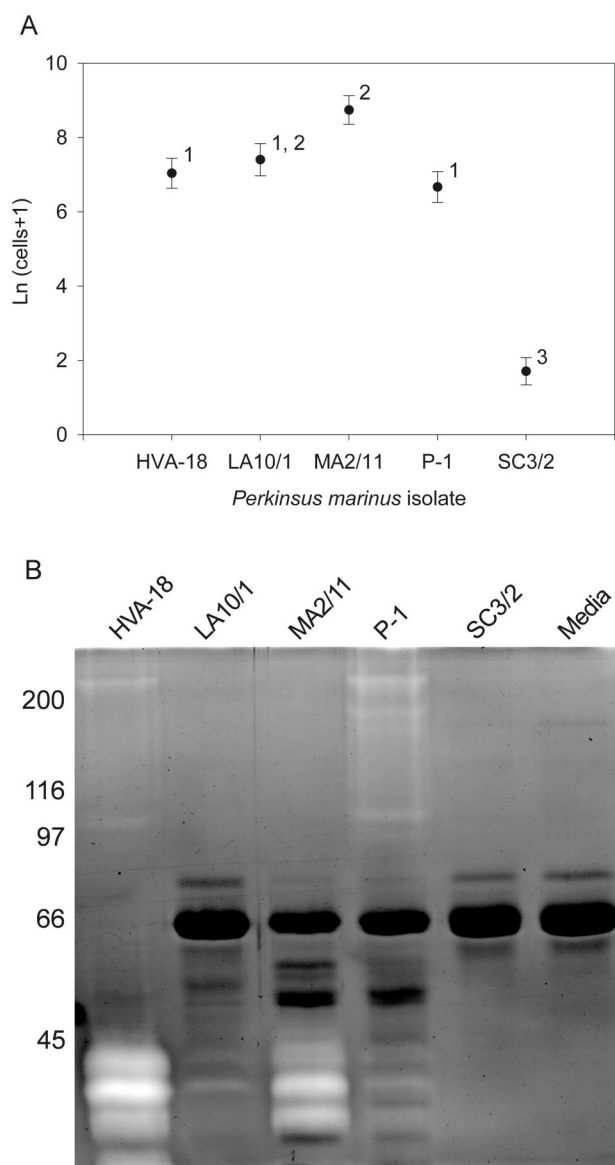


FIG. 1. Infectivity level and secreted protease profiles of selected *P. marinus* isolates. (A) Mean body burden \pm standard error of oysters infected with *P. marinus* isolates. Cells were grown for 3 weeks in Dungan's medium at 27°C in 5% CO₂ and then injected at 10^6 cells per oyster per day on days 1, 3, and 5 into 20 *C. virginica* ME oysters per group. After 4 weeks, the oysters were sacrificed and total *P. marinus* body burden was assessed and log_e transformed for statistical analysis. Groups with different numbers were significantly different ($P < 0.001$). (B) Gelatin zymogram of 20 μ l of culture supernatant from *P. marinus* isolate cultures used in the above infection trial. Nonreducing, 8% acrylamide, 0.1% porcine gelatin SDS-PAGE gels were incubated for 3 h at 37°C in 0.1 M Tris HCl, pH 8.0, and then stained with Coomassie brilliant blue G-250. Molecular masses are in kilodaltons.

than in those grown in ODRP-3 medium (Fig. 2A). Sham-injected controls were all negative for *P. marinus* infection. There were 7, 9, 10, and 3 oyster mortalities in the homogenate, plasma, unsupplemented, and sham injection groups through the course of the experiment. The cause of the mortalities could not be determined due to rapid degradation of the oyster tissues.

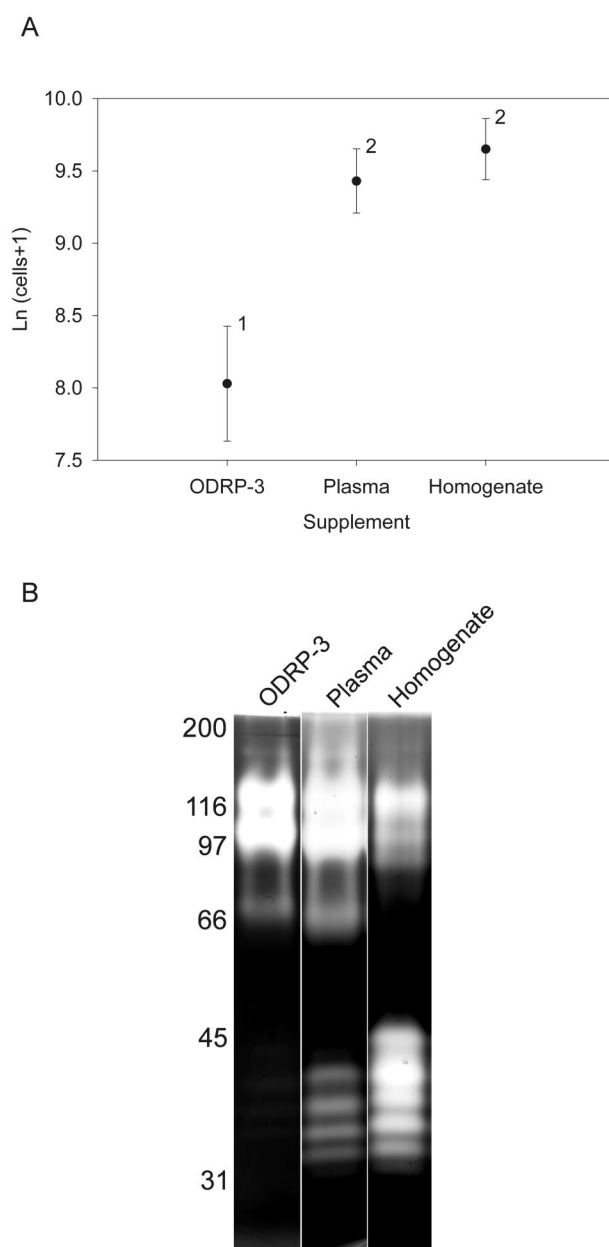


FIG. 2. Infectivity level and secreted protease profiles of *P. marinus* isolate P-1 in plasma and homogenate-supplemented cultures. (A) Mean body burden \pm standard error of oysters infected with *P. marinus* isolate P-1 grown for 4 weeks in ODRP-3 medium without supplementation or supplemented with 0.3 mg of oyster plasma or homogenate/ml at 27°C in 5% CO₂ and then injected at 10⁶ cells per oyster per day on days 1, 3, and 5 into 30 *C. virginica* ME oysters per group. After 4 weeks, the oysters were sacrificed and the total *P. marinus* body burden was assessed and log_e transformed for statistical analysis. Groups with different numbers were significantly different ($P < 0.001$). (B) Gelatin zymogram of 20 μ l of culture supernatant from supplemented and unsupplemented *P. marinus* cultures from the above infection trial. Nonreducing, 8% acrylamide, 0.1% porcine gelatin SDS-PAGE gels were incubated for 3 h at 37°C in 0.1 M Tris HCl, pH 8.0, and then stained with Coomassie brilliant blue G-250. Molecular masses are in kilodaltons.

Oyster tissue homogenate alters *P. marinus* proliferation, size, morphology, and protease expression. Oyster homogenate supplements from all three oyster species resulted in the occurrence of multicellular clusters, which were resistant to disaggregation by pipetting (Fig. 3 and 4). While no shared exterior wall could be visualized, these multicellular clusters were often compressed and were similar in appearance to tomonts. P-1 isolate total cell counts were diminished by oyster homogenate supplementation, regardless of the oyster species (Fig. 3). This effect was dose dependent, with some effect seen even at the lowest supplementation levels. Cells supplemented with *C. ariakensis* homogenate at 1.0 mg/ml appeared to be nonviable, as assessed by neutral red uptake. Supplementation with fetuin was associated with minor diminishment in the number of cells at low and intermediate concentrations and a moderate increase in cell count at the 1.0-mg/ml supplementation level (Fig. 3). Cultures supplemented with FBS behaved similarly to those supplemented with fetuin; however, there was a large increase in proliferation at the highest supplementation levels. When supplemented at 1.0 mg/ml with FBS, the *P. marinus* cell count was approximately 2.5-fold higher than in the ODRP-3 control.

Most multicellular clusters observed in the homogenate-supplemented medium consisted of 5 to 16 aggregated cells. The degree of cell clustering was dose dependent and was typically maximal at intermediate supplement concentrations. The *C. virginica* WA-supplemented cultures had a peak in the number of clusters at 0.11 mg/ml and maintained clusters to the highest dose tested. *C. ariakensis*- and *C. gigas*-supplemented cultures had a peak in cell clustering at the lower supplementation level of 0.037 mg/ml. There was a precipitous decline in the occurrence of these clusters at the 0.33-mg/ml dose for *C. ariakensis* and at the 1.0-mg/ml dose for *C. gigas*. Neither fetuin nor FBS supplementation was associated with formation of multicellular clusters.

Homogenate supplementation from all three oyster species also resulted in a dose-dependent increase in the mean *P. marinus* cell size (Fig. 4 and 5). Cells supplemented at 1.0 mg/ml had an over-threefold-larger diameter than unsupplemented cells. Neither FBS nor fetuin supplementation resulted in a change in the cell size compared with the ODRP-3 control. The calculated cell pellet volume was greater than that of unsupplemented cultures at the highest supplementation levels for FBS-, fetuin-, and *C. virginica* WA-supplemented cultures (Fig. 5B). Both *C. ariakensis*- and *C. gigas*-supplemented cultures had volumes greater than unsupplemented cultures only at the 0.33-mg/ml supplementation level.

A dose-dependent increase in low-molecular-weight protease production was observed, as expected, in cultures supplemented with *C. virginica* WA oyster homogenate (Fig. 6). Homogenates from the *P. marinus*-tolerant *C. gigas* oysters did not induce low-molecular-weight proteases. The *C. ariakensis* oyster homogenate induced low-molecular-weight proteases only at the 1.0-mg/ml supplementation level, and these bands were faint and difficult to visualize on the zymogram. Both FBS and fetuin induced low-molecular-weight proteases at supplementation levels of 0.33 mg/ml and above. Oyster supplement protease activity was seen at approximately 60 kDa in the homogenate-supplemented cultures and media controls, especially at the highest dosage levels. This protease activity was

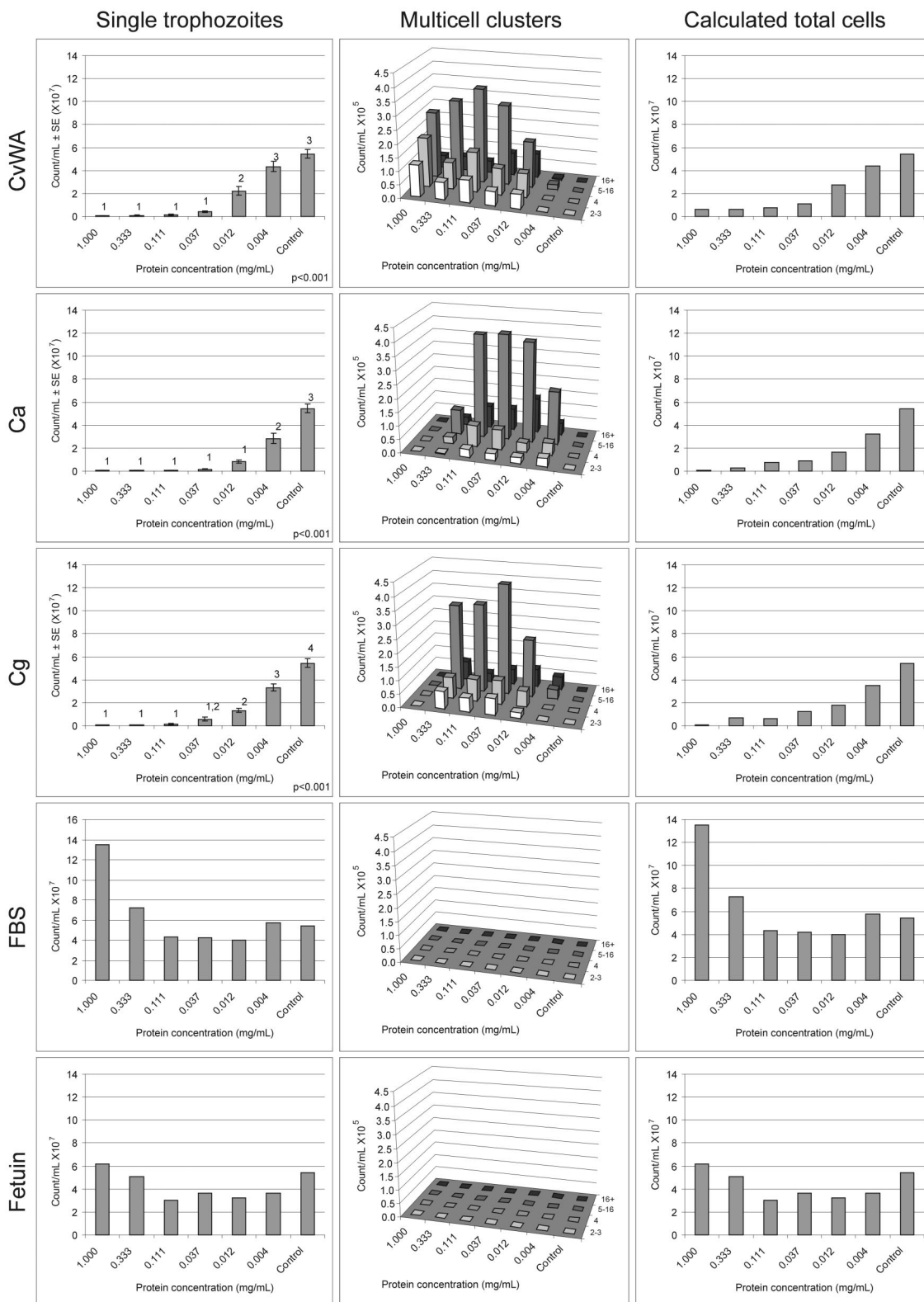


FIG. 3. Mean counts of single trophozoites, multicell clusters, and calculated total cell number (see text) from *P. marinus* isolate P-1 cultures supplemented with a range of doses of tissue homogenate from five *C. virginica* WA (CvWA), *C. ariakensis* (Ca), or *C. gigas* (Cg) oysters, or with FBS or fetuin. Cultures were incubated for 6 weeks at 27°C in 5% CO₂, resuspended by repeated pipetting, and enumerated with a hemacytometer. Counts are the mean of duplicate cultures from each of five separate oysters tested. FBS and fetuin were tested in duplicate. Standard error bars, analysis of variance significance, and multiple comparison results are shown for single trophozoites. Groups with different numbers were significantly different. Note: homogenates from two of five *C. gigas* oysters were not in sufficient concentration to allow 1.0-mg/ml supplementation; therefore, this dose was omitted for these oysters.

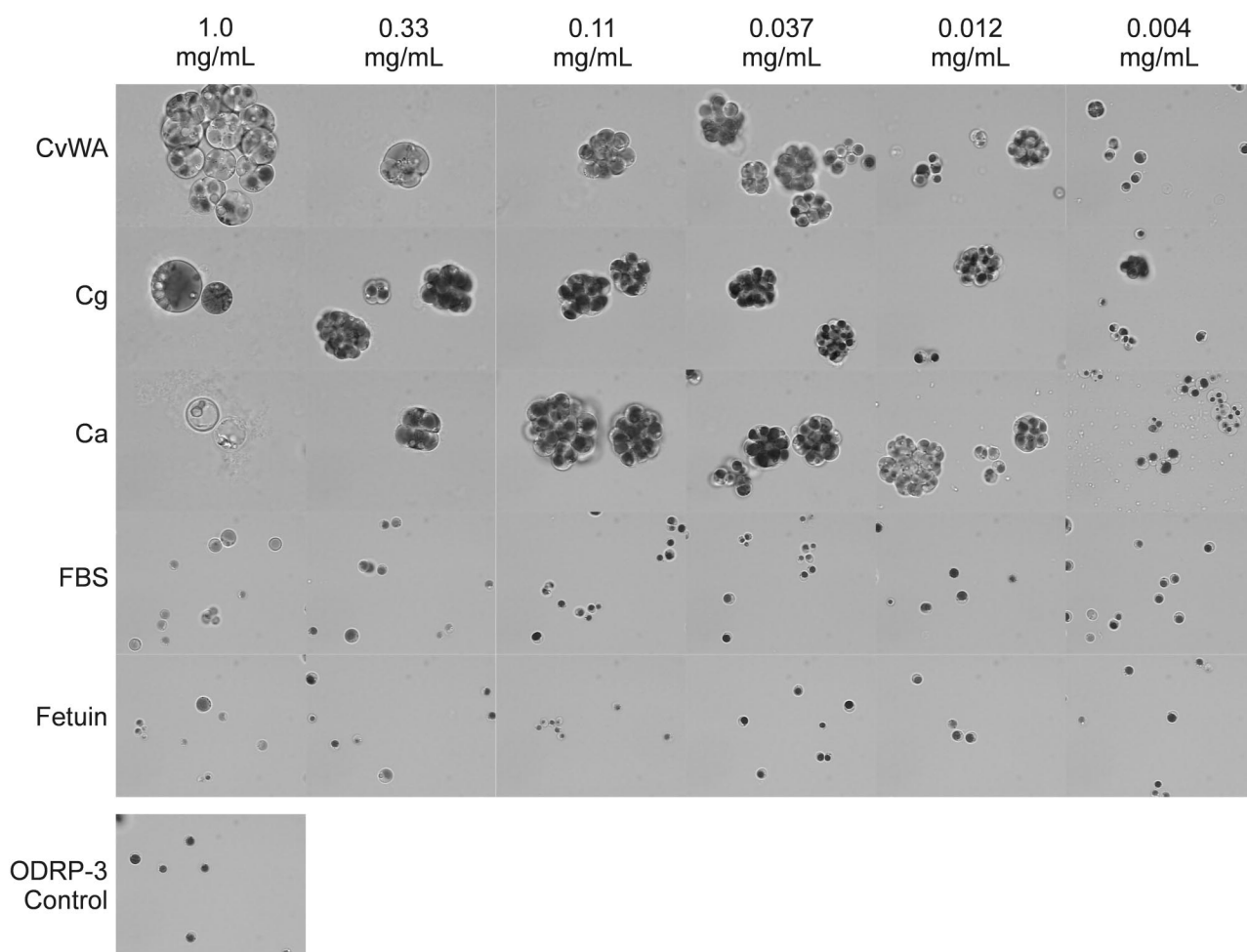


FIG. 4. Light micrograph (neutral red stain; magnification, $\times 1,000$) of *P. marinus* isolate P-1 cultures supplemented with a range of tissue homogenate concentrations from five *C. virginica* WA (CvWA), *C. ariakensis* (Ca), or *C. gigas* (Cg) oysters or with FBS or fetuin. Cultures were incubated for 6 weeks at 27°C in 5% CO₂, resuspended by repeated pipetting, and digitally photographed.

fully inhibited by the addition of 10 mM EDTA to the incubation buffer and is therefore assumed to represent oyster metalloprotease activity similar to that described by Ziegler et al. (42).

Oyster tissue homogenates from different oyster species and populations cause increased infectivity. *P. marinus* isolate P-1 cell cultures were supplemented with 0.25 mg of oyster homogenate/ml from *C. virginica* LA, *C. virginica* WA, *C. virginica* TG, *C. virginica* XB, *C. ariakensis*, and *C. gigas* oysters. Cells from *C. ariakensis*-, *C. virginica* LA-, and *C. virginica* TG-supplemented cultures had a similar mean size of 7.6 to 7.7 μm . Cells supplemented with *C. virginica* XB were slightly larger, with a mean size of 9.0 μm , and those supplemented with *C. virginica* WA were smaller, at 5.2 μm . Unsupplemented cultures had a mean cell size of 4.1 μm . All homogenate-supplemented cultures formed large clusters of cells resistant to disaggregation by pipetting. No clusters were noted in the unsupplemented culture.

All homogenate supplements tested in the infection study resulted in increased body burdens; however, only those oysters infected with cells pulsed with *C. ariakensis* or *C. virginica* LA homogenate had body burdens significantly different than

that of the ODRP-3 control (Fig. 7A). There was one mortality in the *C. virginica* LA group, two in the *C. virginica* XB group, and three each in the *C. ariakensis* and *C. virginica* WA groups. Sham-injected oysters were negative for *P. marinus* infection and experienced no mortalities. Cells grown in the *C. gigas* homogenate-supplemented cultures were not viable at the time of infection, as assessed by neutral red uptake. The cause of this is not known, as concentrations of *C. gigas* homogenate both above and below this level were used in other experiments without loss of culture viability. Gelatin zymograms demonstrated induction of low-molecular-weight protease in the *C. virginica* LA culture only (Fig. 7B). The unsupplemented ODRP-3 culture displayed the typical high-molecular-weight proteases. High-molecular-weight protease activity in the *C. ariakensis* and the *C. virginica* XB cultures was greatly diminished. No protease activity was detected in the media controls.

DISCUSSION

Neither the physical nor the biochemical mechanisms of *P. marinus* infectivity or virulence are understood; however, this parasite can be rapidly attenuated by in vitro culture (16). The

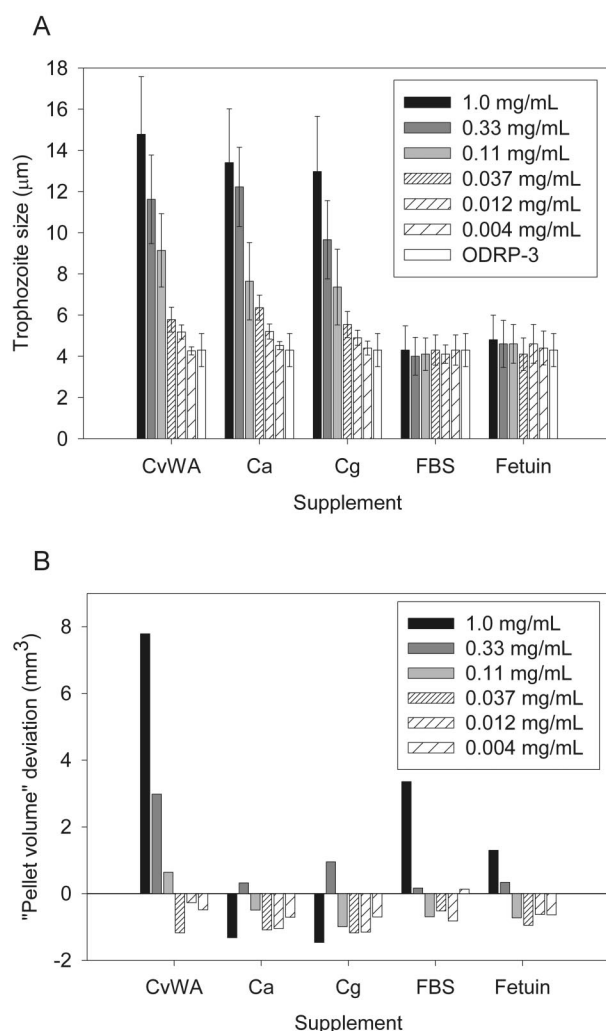


FIG. 5. Supplemented culture cell size and deviation of pellet volume from unsupplemented control cultures. (A) Cell size \pm standard deviation from *P. marinus* isolate P-1 cultures supplemented with a range of tissue homogenate concentrations from five *C. virginica* WA (CvWA), *C. ariakensis* (Ca), or *C. gigas* (Cg) oysters or with FBS or fetuin. Cultures were incubated for 6 weeks at 27°C in 5% CO₂, resuspended by repeated pipetting, and measured at 400 \times magnification using an ocular micrometer. Sizes of 10 trophozoites were measured in duplicated culture wells supplemented with homogenates of five separate oysters per group. *C. gigas* (Cg) measurements included only three oyster supplements at the 1.0-mg/ml dose. (B) Deviation of calculated pellet volume (see text) of *P. marinus* isolate P-1 grown in supplemented cultures from the mean unsupplemented pellet volume.

mechanism of this attenuation is not known, but it may be due to the lack of critical growth, differentiation, or virulence induction factors provided by the host. Previous attempts to restore virulence by passage of in vitro-cultured parasites through a host oyster did not succeed, presumably because of the brief reculture required to produce an inoculum prior to challenge (16). This suggests that attenuation occurs quite rapidly.

***P. marinus* isolates.** Nonclonal *P. marinus* isolates have been shown to vary in virulence, with Atlantic coast isolates being more virulent than those from the Gulf of Mexico (4, 5). In this

study, isolates of *P. marinus* that were chosen based on their assignment by genetic markers into distinct genotypes (37) displayed differences in infectivity, as evidenced by the 4-week body burden (Fig. 1A). The South Carolina isolate, SC3-2, was associated with the lowest level of infection. Eight of the experimental oysters infected with SC3-2 were negative for *P. marinus* infection at week 4, and the highest body burden in any oyster infected with SC3-2 was 96 cells.

It is possible that the small size of the SC3-2 isolate (~ 2 μ m) rendered the cells less likely to be phagocytosed by mantle cavity hemocytes or ingested during feeding. The efficiency of particle filtration by oysters varies directly with the size of the particle (22, 34, 39), and previous studies have demonstrated that ingestion of *P. marinus* is one probable route of parasitic infection (1). While it has also been suggested that hemocytes in the mantle cavity may be responsible for transport of *P. marinus* into the oyster (9, 36), it is not known whether there are size-mediated differences in rates of phagocytosis and transport of *P. marinus* into the oyster by hemocytes. Given these results, there is an indication that *P. marinus* cell size could be a factor in its infectivity.

Supplementation and infectivity. The possibility that cell size is a determinant of infectivity was not supported, however, by the results of the other infection trials. When *P. marinus* P-1 isolate cells were grown in the presence of oyster plasma supplement, the cell size and morphology were similar to those of unsupplemented cultures. The body burden after experimental infection, however, was significantly higher and was statistically similar to that seen with cells enlarged by culture in homogenate-supplemented media. Additionally, despite inducing similar body burdens (Fig. 7A), P-1 cells from cultures supplemented with *C. virginica* WA and *C. virginica* XB homogenates had markedly different mean sizes (5.2 and 9.0 μ m, respectively). The cells with the greatest infectivity were those supplemented with *C. ariakensis* homogenate, and they were very similar in size (mean, ~ 7.6 μ m) to two other groups with lesser infectivities, *C. virginica* LA and *C. virginica* TG. In this latter infection study, infections were initiated in *C. virginica* WA oysters, and it should be noted that the magnitude of the body burdens at 4 weeks was markedly lower than was seen in the earlier trials using *C. virginica* ME oysters. The cause of this is not known, but it is assumed that the *C. virginica* WA oysters may be more resistant to *P. marinus* infection. This is particularly interesting because this population of oysters has been isolated from *P. marinus* for numerous generations (31).

The ability to increase parasite infectivity appears to be a property specific to host plasma and tissue homogenates. Supplementation of cell cultures with FBS in Dungan's medium does not cause the increases in infectivity seen with oyster homogenate or plasma supplementation. The mean 4-week body burden from *C. virginica* ME oysters infected with P-1 grown in Dungan's medium with FBS was 2,327 cells (Fig. 1A), while *C. virginica* ME oysters infected with P-1 grown in plasma and homogenate had mean burdens of 18,975 and 25,311, respectively (Fig. 2A).

Morphology, size, and proliferation. Oyster homogenate supplements were the most effective inducers of morphological changes, altered the protease profiles, and increased infectivity. Supplementation of medium with homogenates from either susceptible or tolerant oyster species resulted in a marked

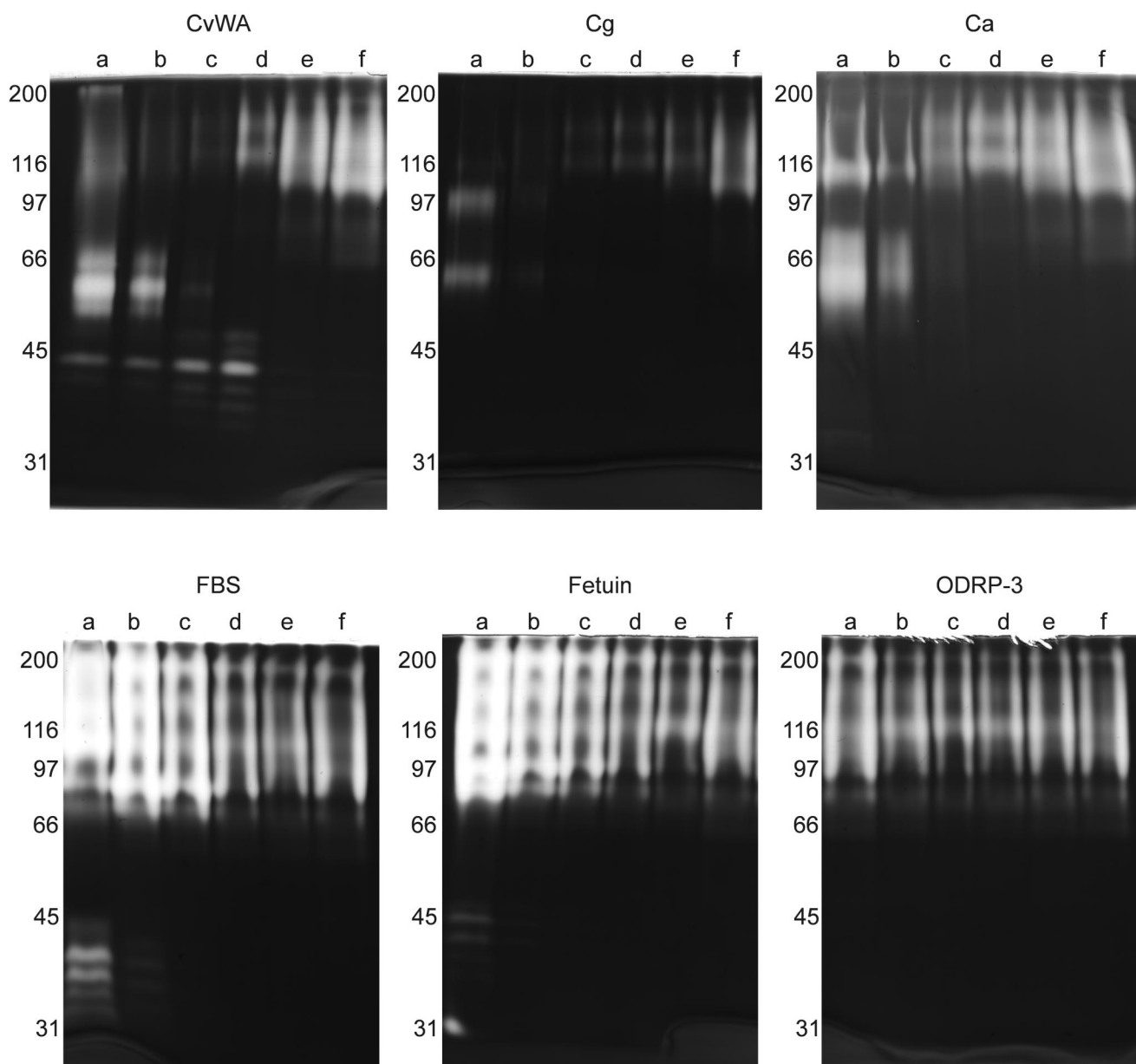


FIG. 6. Gelatin zymogram of 20 μ l of culture supernatant from *P. marinus* isolate P-1 cultures supplemented with 1.0 (a), 0.33 (b), 0.11 (c), 0.037 (d), 0.012 (e), or 0.004 (f) mg of tissue homogenate/ml from *C. virginica* WA (CvWA), *C. ariakensis* (Ca), or *C. gigas* (Cg) oysters or with FBS or fetuin. ODRP-3 is the unsupplemented control. Cultures were grown for 6 weeks at 27°C in 5% CO₂. Nonreducing, 8% acrylamide, 0.1% porcine gelatin SDS-PAGE gels were incubated for 3 h at 37°C in 0.1 M Tris HCl, pH 8.0, and then stained with Coomassie brilliant blue G-250. Molecular masses are in kilodaltons.

reduction in cell proliferation at increasing doses (Fig. 3). At the 1.0-mg/ml supplementation level for both *C. ariakensis* and *C. gigas* homogenates, there was a decrease in cell number below the seeding concentration, with the *C. ariakensis* cells appearing to be nonviable (Fig. 4). At high supplementation levels, the diminution was similar between all oyster species supplements. At the lowest two supplement doses, the suppressive effect was more pronounced in the disease-tolerant *C. ariakensis* and *C. gigas* oysters (Fig. 3). The suppression of proliferation was only associated with oyster product supplementation. Fetuin supplementation caused a minor decrease in

cell proliferation that was consistent at lower supplement doses. Both the 0.33- and 1.0-mg/ml supplementation levels were similar to the ODRP-3 control. FBS behaved similarly, except that there was a large increase in proliferation at the 0.33-mg/ml, and especially at the 1.0-mg/ml, supplementation levels. This is in general agreement with the findings of Gauthier and Vasta (19), who previously described the proliferative effects of FBS and fetuin on *P. marinus* cultures.

Since increasing supplementation levels with oyster homogenates are associated with decreasing cell counts (Fig. 3) and increasing cell size (Fig. 4 and 5A), it was desirable to under-

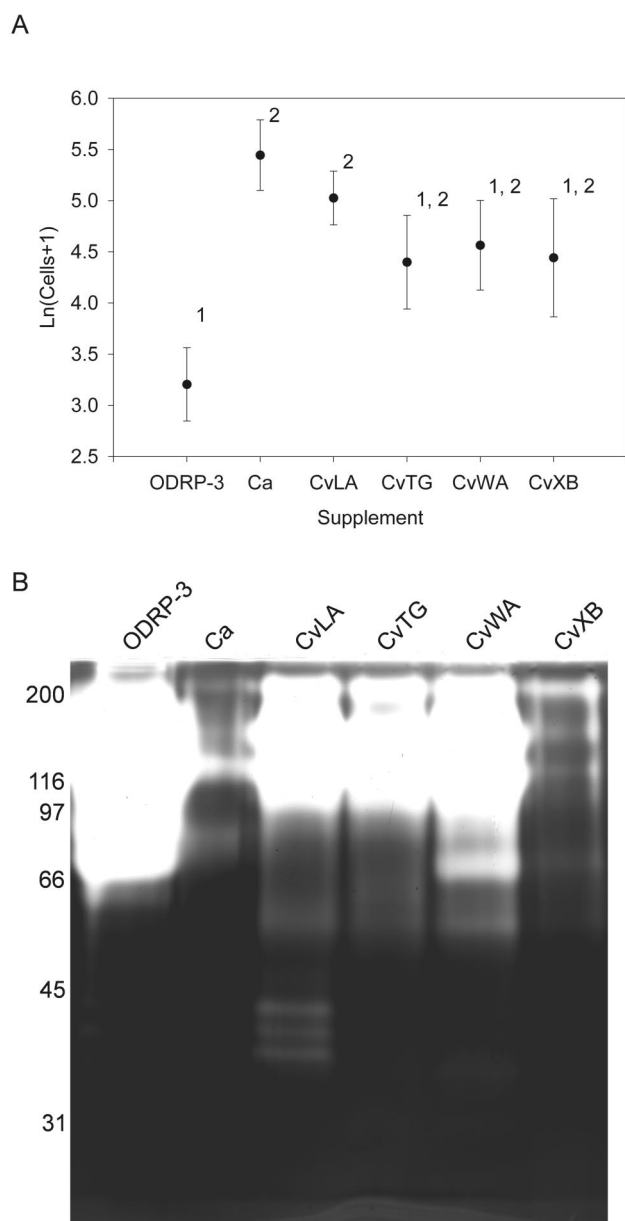


FIG. 7. Infectivity level and secreted protease profiles of *P. marinus* isolate P-1 grown in homogenate supplemented cultures. (A) Mean body burden \pm standard error of oysters infected with *P. marinus* isolate P-1 grown for 4 weeks at 27°C in 5% CO₂ in ODRP-3 medium without supplementation or supplemented with 0.25 mg of homogenate/ml from *C. ariakensis* (Ca) or from *C. virginica* oysters from Louisiana (CvLA), Tangier Sound, Chesapeake Bay (Va.; CvTG), Washington state (CvWA), or from the CROSBreed program (CvXB). A total of 10⁶ cells per oyster per day were injected on days 1, 3, and 5 into 30 *C. virginica* WA oysters per group. After 4 weeks, the oysters were sacrificed and total *P. marinus* body burden was assessed and log_e transformed for statistical analysis. Groups with different numbers were considered significantly different ($P = 0.007$). (B) Gelatin zymogram of 20 μ l of culture supernatant from *P. marinus* isolate P-1 grown for 4 weeks at 27°C in 5% CO₂ in ODRP-3 medium without supplementation or supplemented with 0.25 mg of homogenate/ml as described above. Nonreducing, 8% acrylamide, 0.1% porcine gelatin SDS-PAGE gels were incubated overnight at 37°C in 0.1 M Tris HCl, pH 8.0, with 10 mM EDTA included to limit oyster metalloprotease activity on the zymogram that obscured some of the lower-intensity *P. marinus* serine proteases. The gels were stained with Coomassie brilliant blue G-250. Molecular masses are in kilodaltons.

stand their combined effects using the calculated pellet volumes. The results were not surprising for the FBS and fetuin cultures. Cell sizes did not change over the range of supplementation and there were no cell clusters, and thus, the pellet volumes paralleled the cell counts. However, supplementation with any oyster homogenate was associated with a reduced pellet volume at the three lowest dosage levels (Fig. 5B). At 0.11 mg/ml, the *C. virginica* WA-supplemented cells had a pellet volume in excess of that of the ODRP-3 control. At 0.33 mg/ml, this effect was even more pronounced in the *C. virginica* WA oyster group and was seen in both the *C. gigas*- and *C. ariakensis*-supplemented cultures, despite the continuing decrease in cell count. At 1.0 mg/ml, the *C. virginica* WA culture pellet volume was nearly 8 mm³ larger than that of the ODRP-3 control. At this highest supplementation level, the *C. gigas* pellet volume again fell below that of the ODRP-3 control, as did that of the nonviable *C. ariakensis* culture.

In vivo, *P. marinus* cells range in size from 3.9 to 11.6 μ m, with a mean of 5.5 μ m (35), similar to that elicited by medium supplementation with oyster products. The association of larger cell size with lower replication rate may indicate there is a regulation of mitosis by exposure to host products. *P. marinus* appears to regulate its replication, especially at high infection intensities, such that the parasite burden remains at nonlethal levels (40). This regulation may be based on energy limitations (10). The decreased in vivo replication rate could also be mediated by an ability of *P. marinus* to sense cell density and decrease replication rate accordingly, as has been documented in certain quorum-sensing bacteria (41). This does not seem likely, as previous studies have found that the presence of *P. marinus* cells and extracellular products has a positive effect on the in vitro replication rate (6, 21). Alternatively, this change may represent a partial transition to the prezoosporangium life stage. In fluid thioglycolate medium, trophozoite maturation to prezoosporangia (hypnospores) is associated with thickening of the cell wall and marked enlargement of the vacuole, resulting in cell enlargement to 30 to 80 μ m (35). While this enlargement is considerably greater than is seen in supplemented cultures, it is nonetheless possible that supplementation results in the preliminary phases of transition to this life stage.

The present data suggest that there may be a molecular signal present in all three oyster species tested which regulates *P. marinus* proliferation. Since this effect occurs only with tissue homogenates, the signaling molecule could be a product of tissue degradation. In that case, an increase in the number of *P. marinus* cells during infection and the associated tissue damage from proteolytic activity could signal *P. marinus* to decrease the replication rate and allow the oyster to survive longer in the parasitized state. This possibility is supported by the greater potency of the homogenate effect of the tolerant *C. gigas* and *C. ariakensis* oysters on *P. marinus* cell replication (Fig. 3).

Protease induction. The initial work investigating the induction of proteases by oyster homogenate (29) was largely confirmed by the dose-response experiment, though there was some induction of low-molecular-weight proteases by *C. ariakensis* homogenate, FBS, and fetuin at doses above those assessed in the earlier research. The induction of low-molecular-weight proteases did correlate with infectivity in cultures supplemented with oyster plasma and homogenate (Fig. 3 and

4). It is tempting to correlate increased infectivity with induction of these proteases under exposure to conditions that more closely mimic the *in vivo* environment. The strongest induction of low-molecular-weight proteases and the highest body burdens were seen in the cells supplemented with homogenate. Similarly, the intermediate level of protease production by plasma-supplemented cells was associated with a slightly, though not significantly, lower body burden. Both were more virulent than the ODRP-3 control, which produced little to no low-molecular-weight protease.

The relationship between induction of low-molecular-weight proteases and increased infectivity was not consistent in the other experiments. In the clonal isolates grown in Dungan's medium, the SC3-2 isolate was the least virulent and demonstrated little to no protease activity in the culture supernatant (Fig. 1 and 2). The LA10-1 and P-1 isolate also had lower levels of protease expression in general, and weak low-molecular-weight protease expression in particular, and were more virulent than SC3-2. The HVA-18 isolate had infectivity similar to LA10-1 and P-1, but it had the greatest amount of low-molecular-weight protease expression. The most virulent isolate, MA2-11, had a level of low-molecular-weight protease expression that was intermediate between that of HVA-18 and LA10-1 and P-1. It is interesting that the two Virginia isolates, HVA-18 and P-1, more strongly expressed the series of high-molecular-weight proteases.

When the P-1 isolate was exposed to 0.25 mg of various oyster homogenates/ml, the *C. virginica* LA- and *C. ariakensis*-supplemented cultures were the most infective (Fig. 7A). While the *C. virginica* LA supplementation induced low-molecular-weight proteases, that supplemented with *C. ariakensis* did not. In addition, the *C. ariakensis*-supplemented medium elicited a decreased level of high-molecular-weight protease activity (Fig. 7B). Given these varied results, there does not appear to be a consistent correlation between the *in vitro* induction of low-molecular-weight proteases by homogenate supplementation and infectivity as measured by week 4 body burden.

The origin of the low-molecular-weight proteases and their relationship to the higher-weight forms is not clear. There often appears to be a diminishment in high-molecular-weight protease activity associated with increases in the appearance of the low-molecular-weight forms. It is not known if these changes in the protease profile are due to changes at the transcriptional, translational, posttranslational, or secretory level. Previous research has demonstrated that the low-molecular-weight forms cannot be created from the high-molecular-weight forms by incubation in the presence of host products (29), indicating that the low-molecular-weight forms are likely not originated by proteolytic cleavage of a multimeric form.

The supplementation of *P. marinus* cultures with oyster products from either disease-tolerant or -susceptible oysters modulates cell proliferation, size, and morphology, as well as protease expression and infectivity. Many of these effects cannot be reproduced using common bovine media supplements. None of these varied effects appeared to be linked in all cases, indicating that exposure to oyster products likely causes a complex alteration in *P. marinus* physiology, resulting in a multitude of phenotypic and functional changes.

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